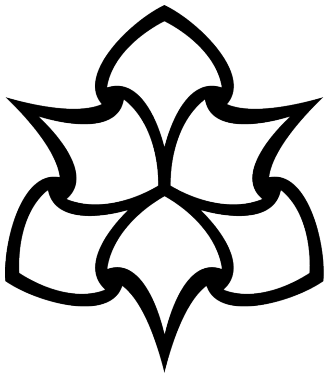

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The effect of dentifrice abrasion on denture topography and the subsequent retention of microorganisms on abraded surfaces

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Statement of problem. Denture surfaces provide hard nonshedding niches for the adhesion and subsequent accumulation of oral microorganisms into denture plaque, which can harbor various potential pathogens linked with oral mucosal lesions and inhalation pneumonia. The initial adhesion is the prerequisite for subsequent biofilm growth, and surface roughness niches facilitate this process by trapping cells. Retained microorganisms are then able to proliferate when the denture is returned to the oral cavity.

Purpose. The purpose of this study was to measure the amount and strength of the attachment of microorganisms to a roughened denture acrylic resin surface. An increase in surface roughness increases the retention of microorganisms and a greater amount of cell-surface contact interface may increase the strength of adhesion and, therefore, retention. Cleaning denture surfaces with brushes and dentifrices can influence the denture surface topography and, therefore, may affect retention.

Material and methods. Denture acrylic resin specimens were abraded to provide different surface roughness. The amount of attachment of *Streptococcus oralis* or *Candida albicans* to these surfaces was assessed by measuring the area of a microscopic field covered by stained cells after 1 hour of incubation. The strength of attachment was assessed with atomic force microscopy, whereby an increasing force was applied to the attached cells until they detached from the surface.

Results. Both bacteria and yeast cells were retained in increasing amounts on surfaces of increasing roughness. Cells were most strongly attached on surfaces whose linear features (scratches) were of comparable size with the cells (the streptococci on the low-abraded surfaces, and the yeast on high-abraded surfaces).

Conclusion. Analysis of findings reveal that even small abrasions may enhance retention on denture surfaces and reduce surface cleanability. The strength of attachment instead of the amount is more important in terms of surface hygiene. (J Prosthet Dent 2014;■:■-■)

Clinical Implications

Denture hygiene regimes should aim to remove microbial contamination while minimizing the amount of abrasion to the denture surface to reduce susceptibility to the proliferation of denture plaque that may harbor potential pathogens and/or lead to the onset of denture stomatitis.

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Denture acrylic resin is softer than tooth enamel and as such is more susceptible to abrasion.[1] If inappropriate products are used to clean the denture, for example, dentifrices intended for teeth, then the acrylic resin will become scratched.[2] Surfaces with increased roughness are better able to retain both microorganisms[3-5] and organic material,[6] thus reducing cleanability. The application of increased force then is required to clean the surface, which results in increased surface wear. Thus, a cycle of wear and soiling that is already well recognized in other applications[6] is initiated but that has received relatively little attention in prosthetic dentistry.[2]

The literature on the effect of surface roughness on the retention of oral microorganisms on surfaces is significant, but some difficulties can be encountered in comparing results from different studies because of the number of variables used. These include different methods of producing and characterizing the modified,[7] different experimental conditions[8,9] and different microorganisms of different dimensions.[10-12] Microbial cells will likely be retained within surface defects (such as scratches or pits) of comparable dimension with the cells themselves,[13] but the relationship between these 2 parameters has not been explored in prosthetic dentistry. Methods used to assess the amount of retention also should be differentiated and measured, for example, by assessing the numbers of retained cells or coverage of the surface by cells, and the strength of attachment, the latter perhaps being the more pertinent phenomenon.[14] No studies could be identified that compared these 2 phenomena.

The aim of this study was to investigate whether the abrasion of denture acrylic resin caused by dentifrice application affects the retention of microorganisms and thus surface cleanability and hygiene. Two microorganisms were used: *Candida albicans*, generally deemed to be a significant etiologic agent in denture stomatitis,[15] and *Streptococcus oralis,* an early colonizer of hard oral surfaces.[16] Two assays were used: a "retention assay," which compared the retention of cells on surfaces after a 1-hour exposure, and a standardized rinsing procedure, which gives an indication of the "amount of attachment," and a "strength of attachment assay," which applies an increasing force onto attached cells to remove them.

MATERIAL AND METHODS

Denture material (polymethylmethacrylate based and filled) was used in specimens 20 mm^2 in area and 5-mm thick. These were abraded in a defined manner by using 800 strokes with blocks rotated 180 degrees after 400 strokes under 2.9 N force[17] to provide linear, approximately parallel, abrasions. The abrasive used was a toothpaste slurry, with the dentifrice selected to provide different degrees of abrasion as follows: Colgate Luminous (Colgate Ltd) was used neat (undiluted) to provide high abrasion (HA); medium abrasion (MA) used Colgate Total Whitening (Colgate Ltd) 25:40 paste-water ratio; and low abrasion (LA) used Colgate cavity protection (Colgate Ltd) 25:40 paste/water ratio. A non-abraded control was included.

White light profilometry

A MicroXAM (phase shift) surface mapping microscope (ADE; Omniscan) with an analogue to digital (AD) phase shift controller (Omniscan) was coupled with an image analysis system (Mapview AE 2.17; Omniscan) to characterize the surface.[18] Analysis was carried out in the EX mode, with 10 different fields examined for 3 replicate specimens of each abraded surface. Surface maps were generated, along with surface profile and roughness values (S_a, average deviation of the roughness irregularities from the surface mean center line; and S_q, standard deviation of the distribution of the surface peak heights), at a magnification of ×101.6 (86.14 × 64.07 μm). Measurements of representative feature width and depth were made.

An atomic force microscopy (AFM) (Explorer; Veeco Instruments Ltd) was operated in contact mode with silicon nitride pyramidal-shaped tips, with a nominal spring constant of 0.05 N/m. The averages of R_a (average deviation of the roughness irregularities from the surface) and R_q (standard deviation of the distribution of the surface peak heights, also known as RMS) values for the surface profile were obtained from 3 linear scans taken across individual specimens over 50-μm^2 fields[19] with a scan rate of 10.03 μm/s and 300 pixel resolution.

AFM data also were used to determine whether the surfaces could be used in the subsequent strength of attachment assays, particularly for the yeast cells. Because *Candida albicans* cells can measure up to 5 μm and the z height limit of the AFM is approximately 6 μm, too rough a surface would result in the AFM being unusable either to visualize the cells or assess the strength of the attachment. The preparation of cells was carried out for both retention and AFM assays by using sterile distilled water because the use of saline or phosphate buffered solutions interfered with the strength of attachment and staining procedures after the retention experiments.

Stock cultures of *C albicans* NCYC 1467 (also known as GDH 2346) were stored at −80°C. Subcultures were prepared on Sabouraud agar (Lab M) before use and were maintained at 4°C. New stocks were prepared from the frozen stock cultures every 4 weeks. In preparation for assays, stock cultures were inoculated onto Sabouraud agar and incubated at 37°C for 24 hours. A single colony of *C albicans* then was inoculated into 100 mL of Sabouraud broth (Lab M), which subsequently was incubated at 37°C for 24 hours in an orbital shaker. Cells were harvested by centrifugation, washed twice in sterile water, then resuspended in sterile water to an optical density of 1.0 at 540 nm, which corresponds to a standardized cell suspension that contains approximately $7.8 \pm 0.18 \times 10^4$ cfu/mL.

Stock cultures of *S oralis* NCTC 11427 were stored at −80°C. Subcultures were prepared before use and were maintained at 4°C. New stocks were prepared from the frozen stock cultures every 4 weeks. In preparation for assays, stock cultures were inoculated onto blood agar and incubated at 37°C for 48 hours under anaerobic conditions. A single colony of *S oralis* then was inoculated from a blood agar plate into 100 mL of BHI broth (Lab M) and incubated for 18 hours, without shaking, at 37°C in an anaerobic cabinet. Cells were harvested by centrifugation (3600*g* for 12 minutes) and washed once in 10 mL sterile distilled water. The resultant standardized cell suspension was adjusted to an optical density of 1.0 at 540 nm, which corresponds to a concentration of $1.32 \pm 0.81 \times 10^9$ cfu/mL.

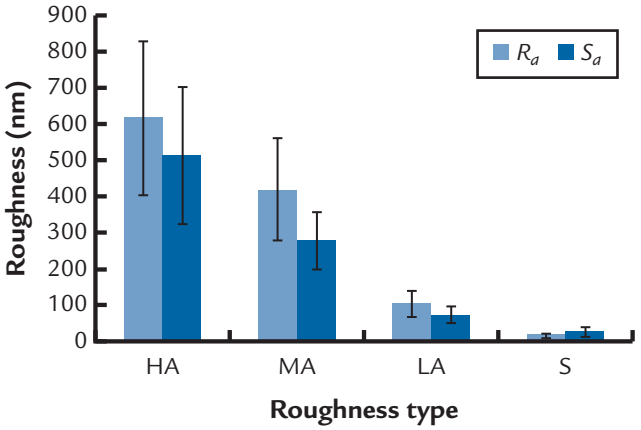
Standardized cell suspension (150 mL) was added to a large (135-mm diameter) petri dish that contained 3 replicates of each of the test materials, which then was incubated for 1 hour at room temperature (25°C ±2°C) without agitation. After 1 hour, the test materials (with adherent cells) were removed and gently rinsed once with 5 cm^3 distilled water, with a water bottle with a 3-mm nozzle held at a 45 degree angle for 3 seconds,[20] which allowed the flow of liquid over the surface and which removed loosely adhered cells. The surfaces then were tilted to drain excess liquid and left to dry in a class II laminar flow cabinet.

Cells retained on the surfaces were stained with acridine orange (Sigma) (0.03% in 2% glacial acetic acid) (BDH), and the surfaces were rinsed and dried before examination with epifluorescence microscopy (×400) (Nikon Eclipse E600; Nikon UK Ltd). Ten fields of each replicate surface were examined. The percentage of an area of each microscopic field was calculated by using cell F software (Olympus Soft Imaging Solutions); the number of cells in each field also was counted. The experiment was repeated.

To determine the strength of attachment, a known force was applied to the attached cells with the AFM cantilever tip, and this force was increased until cells were dislodged. These measurements usually focus on displacement forces applied perpendicular to the substratum surface. By deliberately increasing the perpendicular tip–surface force, the scanning pattern of parallel lines of the AFM tip can be used to displace weakly attached particles and can be used to determine the lateral force of interaction by using the calculations of Deupree and Schoenfisch.[21] On the abraded surfaces, the strength of attachment assays were carried out either along or across the linear features.

One hundred microliters of cells were applied to the test surfaces and dried for 1 hour under a microbiologic, class 2 laminar flow hood and for an additional 23 hours at room temperature in a sterile container. The AFM was operated in the contact mode, and measurements were carried out at a rate of 10.03 µm/s at a scan size of 50 μm^2 (*C albicans*) and 10 μm^2 (*S oralis*). Substrata with dried cells were placed on the AFM stage, and a dry scan of the sample was taken to ensure the presence of cells in the area of analysis. One hundred microliters of high performance liquid chromatography (HPLC) grade water (BDH) were placed on the specimen, and the AFM laser was realigned. The cantilever was brought into contact with the surface under the water, a measurement of the force applied to the cantilever was obtained from force-distance curves,[19] and a perpendicular applied force was calculated.[19,20] Replicate substrata were used, which enabled 4 detachment experiments per surface treatment. After each scan, the remaining cells were counted manually in the field of view, and the counts were plotted as a percentage value of the initial count against the lateral force applied.

In preliminary work, for *C albicans* on the smooth surface and LA surfaces, the cells were lifted after wetting straight from the surface within 2 scan passes. Therefore, for subsequent experiments, after the first wet scan, cells were redried onto the surface, which then was rescanned. This redrying process was repeated for every scan. The coverage of surfaces with 4 different surface roughness types was compared for the percentage of either the *C albicans* or *S aureus* cells retained. Because the data did not fit a normal distribution, these data were initially analyzed by using a Box-Cox transformation, which suggests that normalization of the data with a log

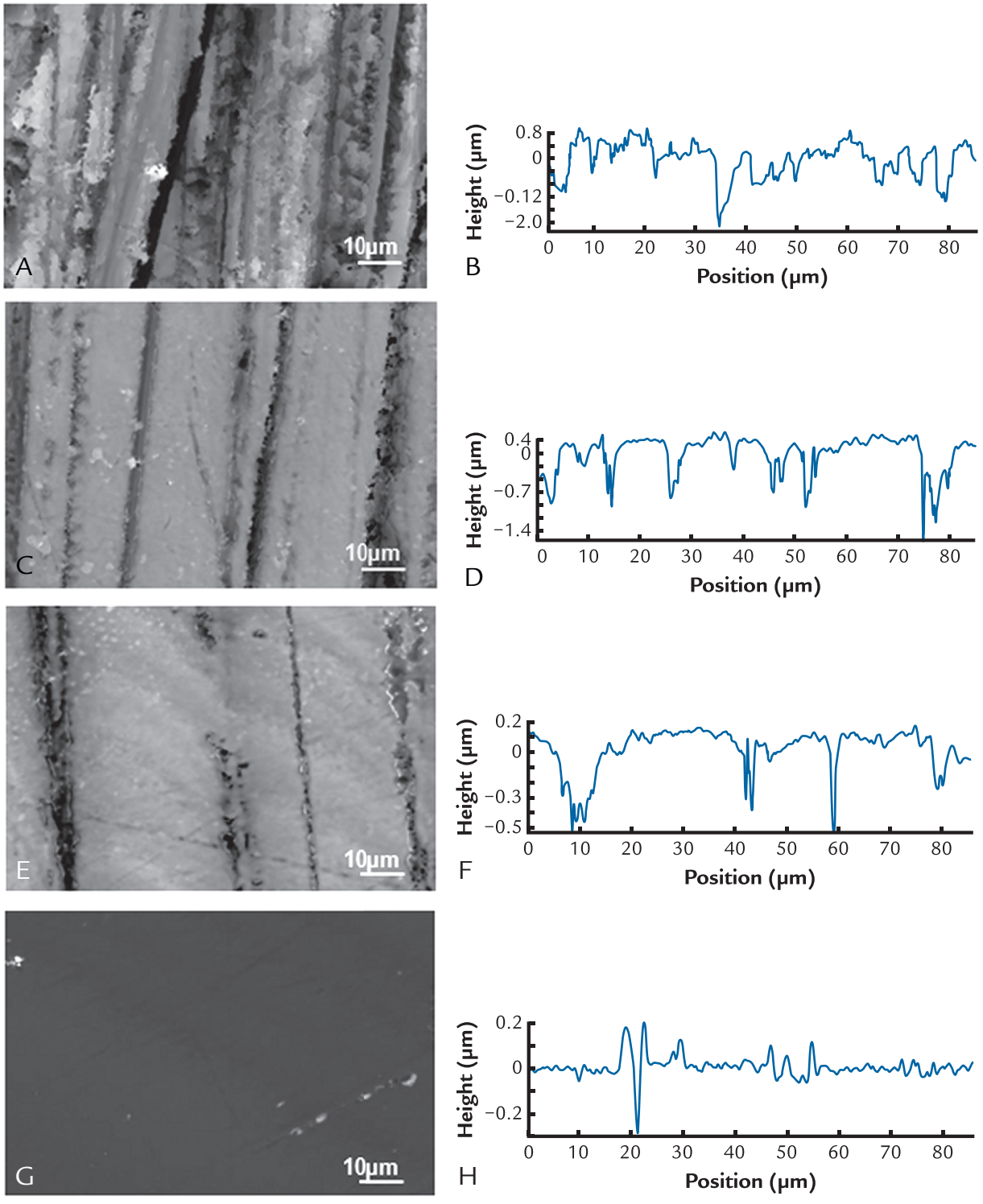


1 Surface roughness values (S_a and R_a) for abraded substrata with white light profilometry (S_a values) and atomic force microscopy (R_a values). S_a, average deviation of the roughness irregularities from the surface mean center line; R_a, average deviation of the roughness irregularities from the surface; HA, high abrasion; MA, medium abrasion; LA, low abrasion; S, smooth nonabraded (control).

transformation would be appropriate. However, zero values also were present within the data sets from microscopic fields that were randomly chosen but contained no retained cells. To accommodate these zero values in the data, these values were treated as censored, the censoring point being the smallest recorded value in the data set. A likelihood ratio was applied to the data sets to test for any differences in the mean (percentage) coverage across the 4 different surfaces. A simple Bonferroni adjustment was made to provide an approximate family-wise 95% confidence level when the intervals for the comparison with the control level were calculated.

RESULTS

For both the white light profilometry (WLP) and AFM (Fig. 1), all roughness parameters increased with increasing

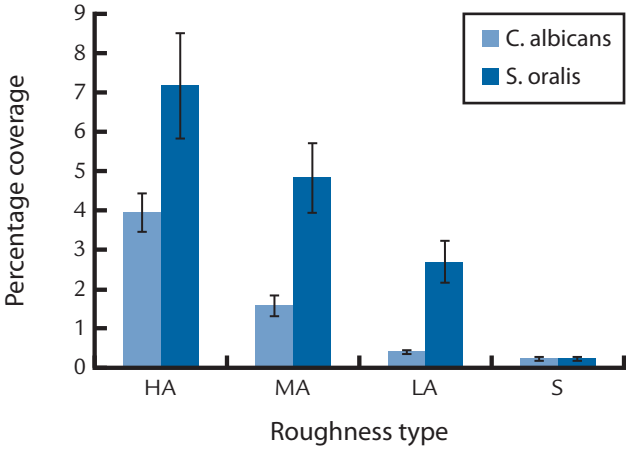


2 Image and surface profiles. A, B, Highly abraded surface. C, D, Medium-abraded surface. E, F, Low-abraded surface. G, H, Smooth, nonabraded surface, showing substratum topography and features.

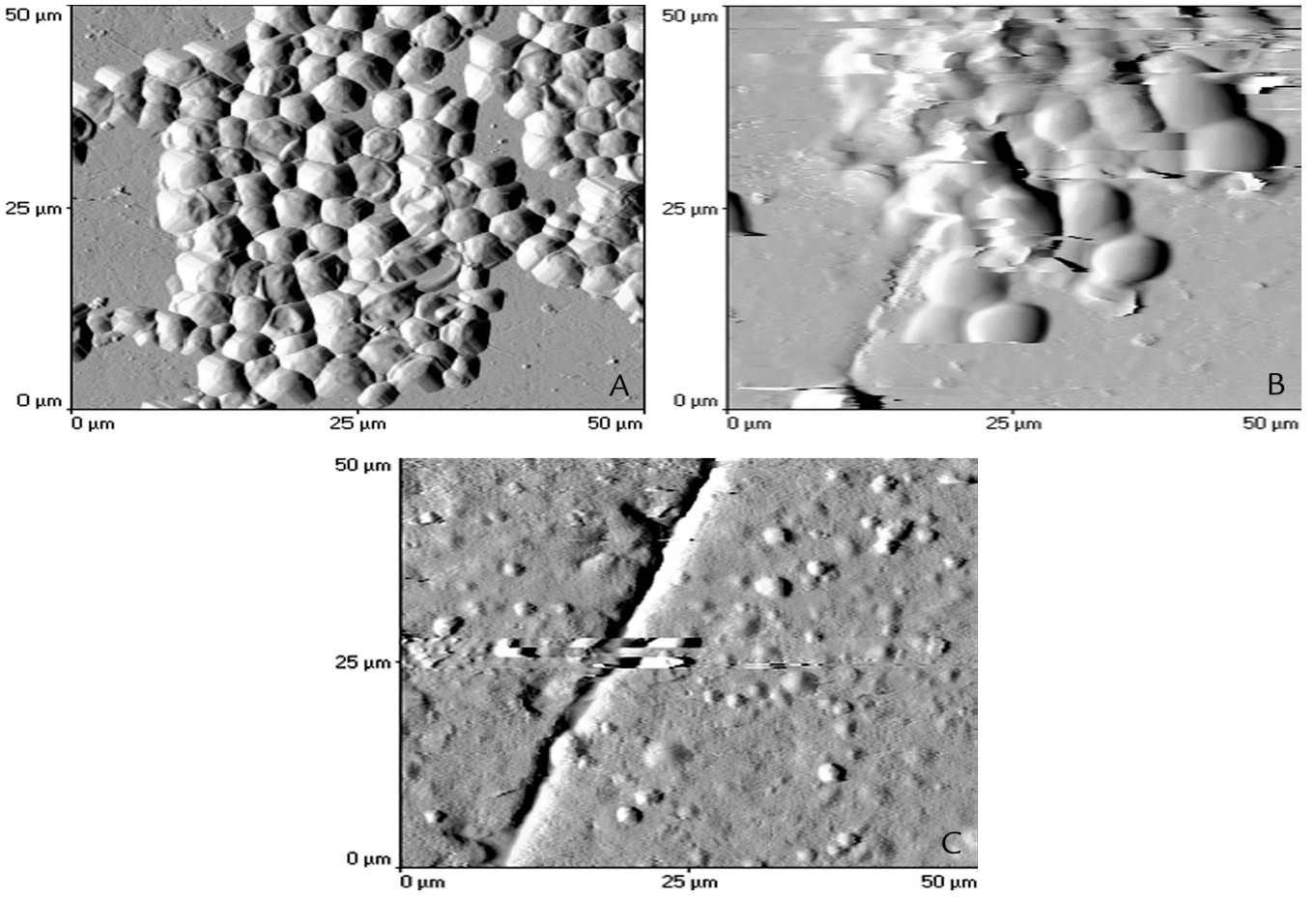
abrasion. Differences in the S_a/R_a and S_q/R_q data were greater for the rougher surfaces. WLP images revealed linear features (scratches) of decreasing number in a given area, from HA to LA surfaces. The profiles revealed the width and depth of these scratches (Fig. 2B, D, F, H). The maximum width was similar for all abraded surfaces at 30 to 35 µm, but approximately half that value for the few scratches noted on the control surfaces. Scratch depths ranged from 0.003 µm to well over 1 µm on all other surfaces. The number of scratches was higher with increasing abrasion. Fewer deep abrasions were apparent on the LA surfaces. For all abraded surfaces, smaller defects were evident within larger scratches.

The smaller AFM probe provided additional data. On the LA surface (data not shown), only occasional scratches could be seen because, on this scale, they were widely separated. These were 1- to 3-µm wide, with an average depth of 1.3 µm. The MA surfaces presented more scratches, which ranged in size from 0.5 to 7 µm in width with an average depth of 2.3 µm. The HA surfaces showed scratches with the greatest average depth (3.3 µm) but also presented some features narrower (0.5-5 µm) than those observed on the MA surfaces.

By using retention assays for both microorganisms, cells were retained in greatest numbers on the HA surfaces, followed by the MA and LA surfaces and smooth surfaces (Fig. 3). The standard deviation was greater for rougher surfaces, especially for the



3 Coverage of high abraded (HA), medium abraded (MA), low abraded (LA), and smooth (S) surfaces by *Candida albicans* and *Streptococcus oralis* (n=30).



4 *Candida albicans* cell removal with cantilever tip moving across surface features. A, Dry scan. B, Second scan under water with increased force (3.7 nN). C, Fourth scan under water with increased force (4.3 nN).

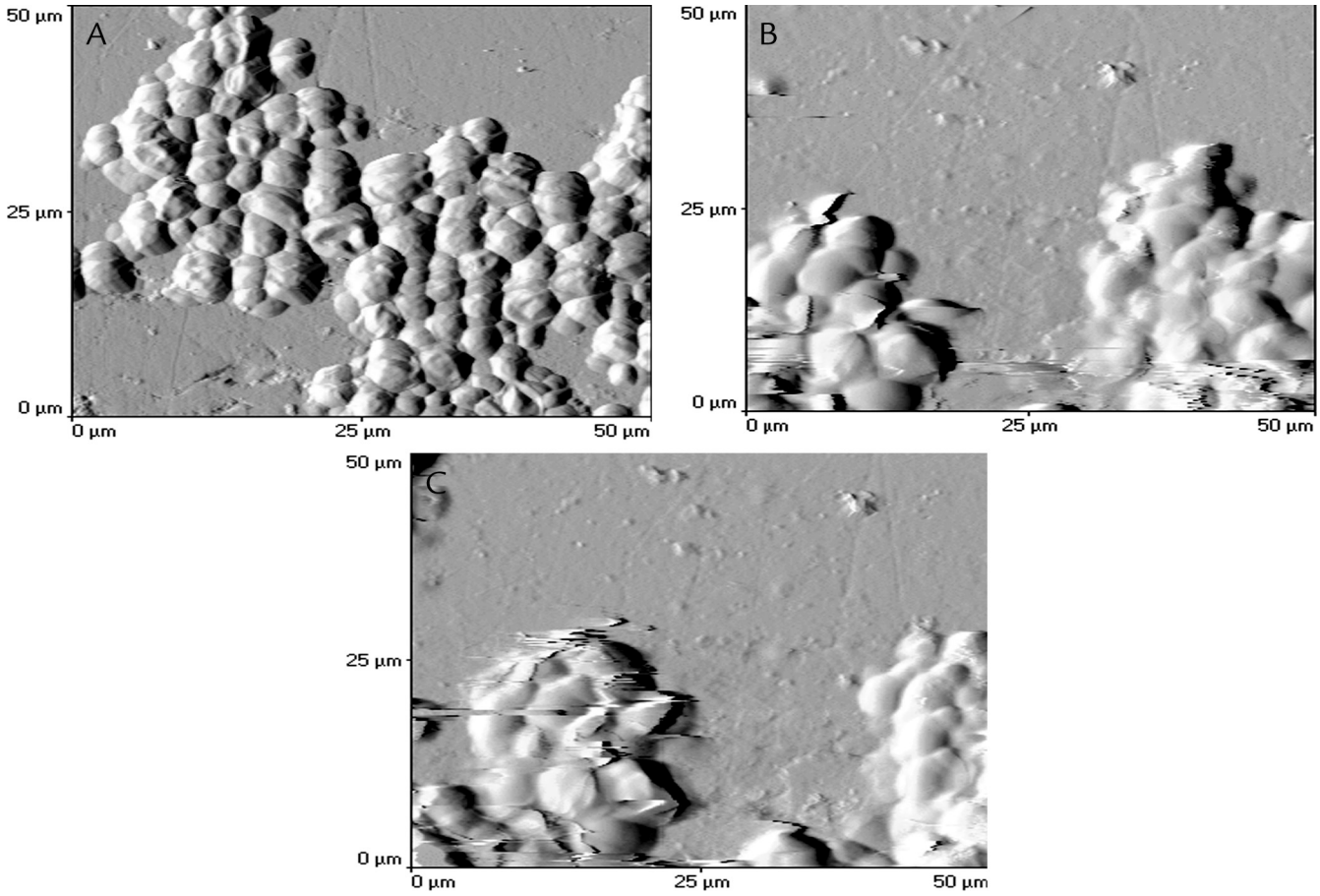
bacteria. Coverage by bacteria is higher than by yeast (as it was in the initial inoculum). Cell counts also were made in each field for *C albicans* and were comparable with those of the coverage data. However, some differences due to different cell sizes (budding cells, pseudohyphae) produced similar counts but different (higher) coverage data (results not presented). The numbers of bacteria were too high to count.

Very few yeast cells were retained on the smooth surface, in spite of the application of low forces (<2.2 nN). *C albicans* was more easily removed from the LA surface when the tip was moving across the surface features (Fig. 4) instead of along them (Fig. 5). When the strength of attachment was assessed by applying force across the scratches on the abraded surfaces (Fig. 6A), an increase in abrasion treatment (low to high) size increased the strength of cell attachment. In all cases, the first scan removed the majority of retained organisms. When the strength of attachment was assessed by applying force along the scratches (Fig. 6B), cells persisted on the LA surface for up to 7 scans. Attachment was higher on the MA and particularly on the HA surfaces when the force was applied across the features. Thus,. overall, the yeast cells were harder to remove when force was applied along the scratches, and cells were more strongly retained on the more abraded surfaces.

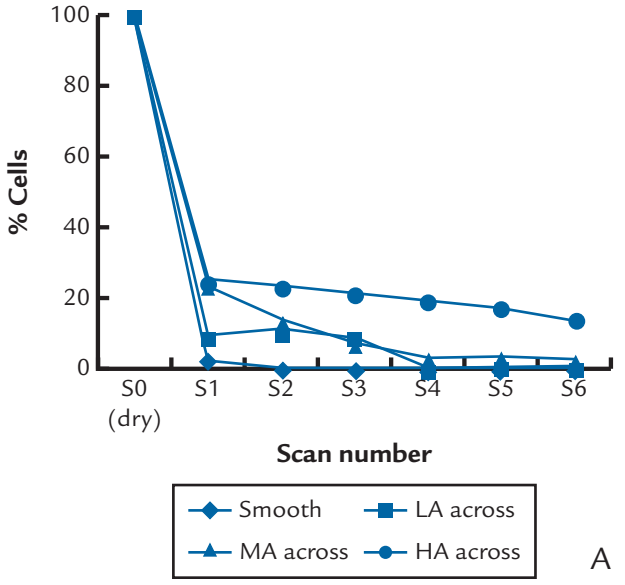
As soon as *S oralis* was immersed in water, the cells seemed to swell (Fig. 7), although some removal did occur with increasing numbers of scans. In contrast with *C albicans* (Fig. 6) on the smooth polymethylmethacrylate surfaces, *S oralis* cells were harder to remove (Fig. 8), with cells still attached to the surface at scan 7. When the strength of attachment was assessed by applying force across the scratches (Fig. 8A), cells were strongly attached to LA and MA surfaces, with 50% of the initial cell number remaining after 7 scans. In contrast, on the HA surfaces (roughest), most of the bacteria were removed after only 3 scans. When the strength of attachment was assessed by applying force along the surface features (Fig. 8B), cells could not be removed from the LA surfaces. More cells remained on the MA surfaces than on the HA surfaces, which indicated that the bacterial cells, considerably smaller than yeast, were most strongly held within the smaller scratches on the LA surfaces.

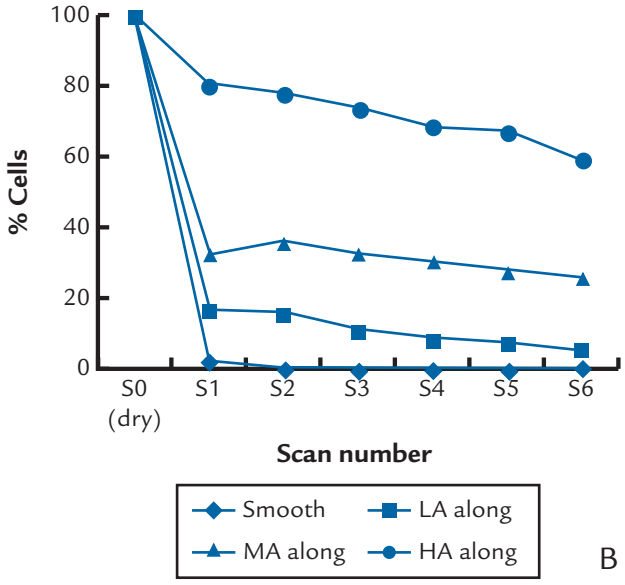
DISCUSSION

In an attempt to simulate wear caused by the use of dentifrice, several methods have been used to characterize denture acrylic resin surfaces that have been abraded in a standardized manner. Increasing abrasion increased



5 Cell removal with cantilever tip moving along surface features. A, Dry scan. B, Second scan under water with increased force (3.0 nN). C, Seventh scan under water with increased force (9.7 nN).





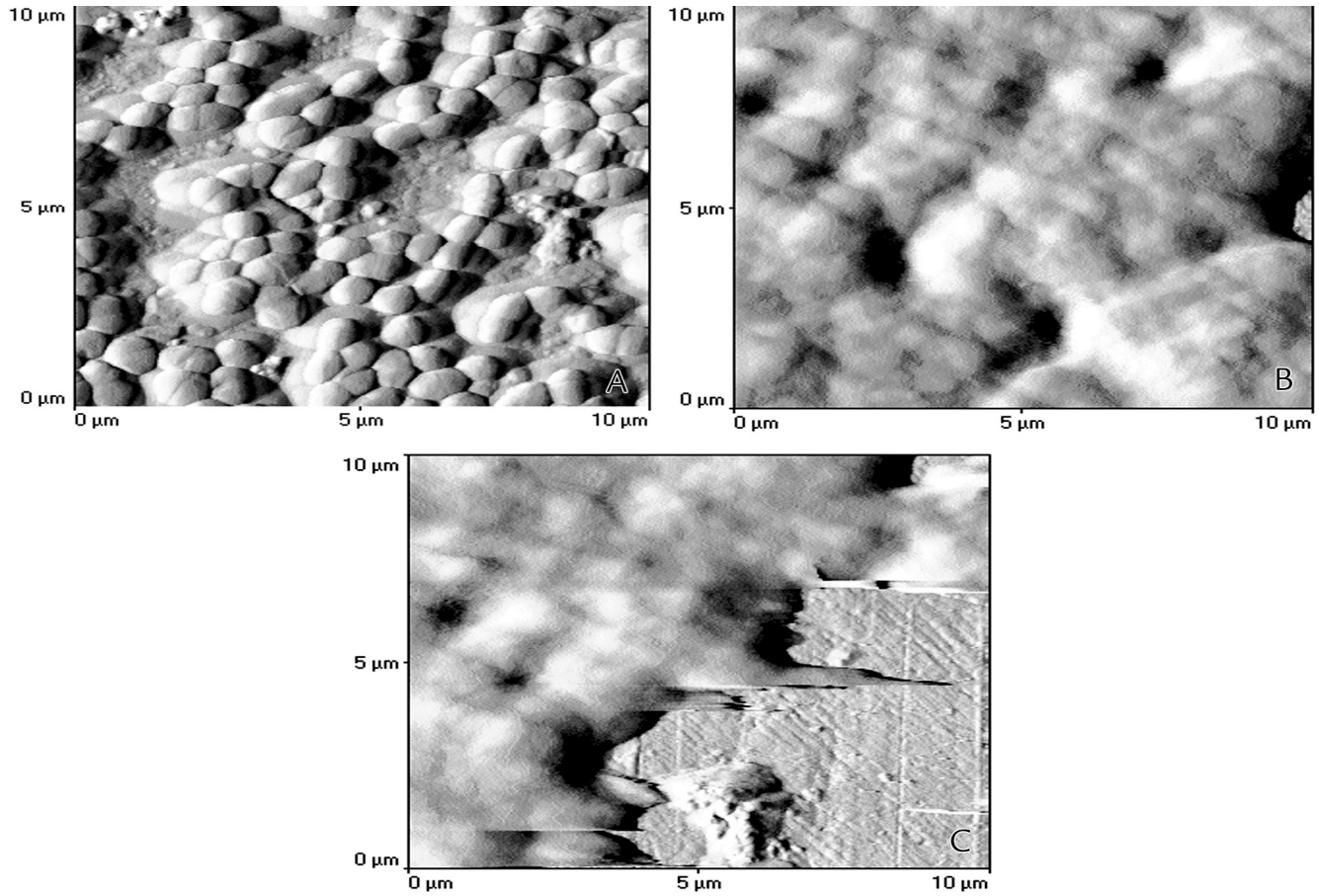
6 Strength of attachment measurements. A, Carried out across surface features. B, Along surface features with *Candida albicans* on surfaces of differing topographies by using increasing number of scans (S) with increasing applied force. On the smooth surface, very few yeast cells were retained. *C albicans* was more easily removed from LA surface (4 scans) when tip was moving across surface features (B compared with E) (7 scans). Increase in abrasion treatment (low to high) increased strength of cell attachment. Cells were retained more strongly on MA and HA surfaces with force applied along features. LA, low abrasion; MA, medium abrasion; HA, high abrasion.

all roughness parameters, which found the susceptibility of denture acrylic resin to abrasion and an increased propensity for subsequent increased plaque accumulation[22] and more vigorous cleaning. Although the authors are aware that many other factors influence the initial attachment of cells to the surface, pH, cell species, surface charge (of both cells and the surface), production of adhesions, organic material, and co-adhesion of cells to name a few, this study aimed to provide a preliminary look into the effect of surface roughness on cell attachment and retention before other variable are further explored.

The actual numeric values for roughness obtained by WLP and AFM were similar. The differences in probe size, numbers of replicate measurements made, and size of area analyzed as well as variability among individual features on the abraded surfaces are factors that may help account for differences. However, this study was not meant to simulate reproducible linear features exactly[19] but to standardize the abrasion process to produce substrata of sufficiently different topographies to enable comparison. The variability observed between feature sizes in this study indicates that, if the size of surface features to interaction is to be related to microorganisms, then more data should be obtained to describe the profile, width, and height of a number of replicate features on these surfaces, which will enable a more comprehensive characterization instead of relying on statistical indicators of the deviation of a profile.[23]

Data were obtained regarding the dimensions of linear features (scratches) created by abrasion, but findings indicated that more data should be obtained. AFM data revealed that the depth of scratches increased with increasing abrasion. The range of scratch widths was wide, but measurements among the 3 abraded surfaces with either AFM or WLP did not change to any large extent. Determining whether increased width at the top of the scratches on the more abraded surfaces was not possible because of a lack of data. Because more scratches on the more abraded surfaces were observed, the surfaces may be more difficult to clean because of increased feature frequency, depth, and a more irregular profile.

The probe size of a given profilometer will affect the sensitivity of the equipment used, detail of the profile generated, and, hence, the data obtained.[7] Equipment that provides the

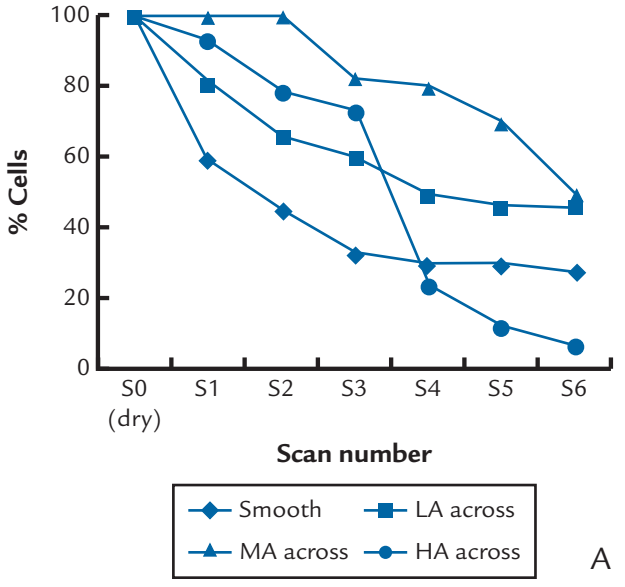


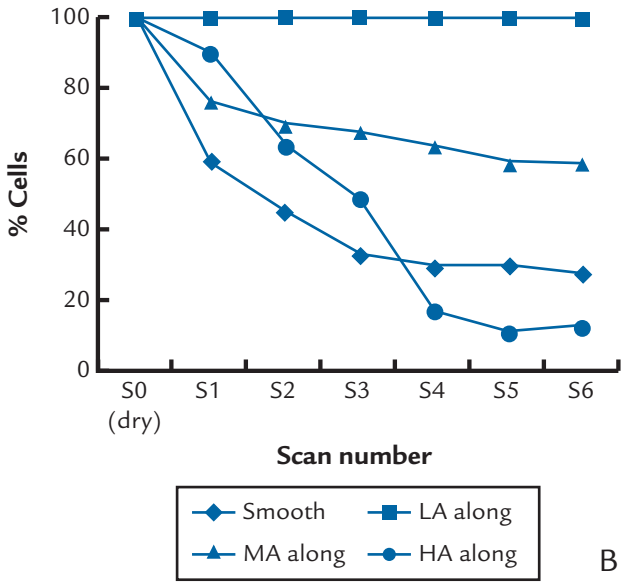
7 A, Dry scan of *Streptococcus oralis* on surface. B, C, With increasing (wet) scans, cells were removed, but some extracellular material is apparent when cells were wetted.

most sensitive measurements at the dimension of the microorganism of concern, in this case, AFM, should be selected as appropriate. However, both WLP and AFM revealed that topographic features that are particularly relevant to the retention and attachment of microorganisms (features of micrometer dimension) are often present within larger features, in this case, scratches.

For both yeast and bacterial cells, an increase in surface roughness as indicated by the R_a or S_a value increased the numbers of cells retained. In addition, at higher coverage/cell number levels, the data would likely provide an underestimation of the total contamination due to cells being beneath others, particularly on the more abraded surfaces with deeper features. This phenomenon could be explored further by using confocal microscopy, along with investigations regarding the effect of shear on attachment and retention. Little salivary flow occurs on the occluded denture fitting surface, and plaque accumulates in this relatively stagnant environment; however, flow cells enable additional exploration of the interactions that occur between cells and the surface. The number of cells retained, however, is of little consequence if they are easily removed and have merely settled into the crevices. It is the strength of the cell attachment data that is of more value, which suggests that cells are more strongly retained on surfaces where feature size approximates cell size. The differences between the bacteria and the yeast in terms of strength of attachment can be related to the cell size relative to feature size; hence, the area of interaction between cell and feature.[12] When cells were firmly retained, the application of a force across a linear feature was more effective at dislodging the cell than the application of a force along the feature, presumably because of the resistance provided by the opposing wall of the feature. Differences between the across and in-direction measurements were most noticeable on the surfaces that had features similar to the size of the cell being investigated, that is, the LA surfaces for *S oralis* and the HA surfaces for *C albicans*. The requirement that the cells be dried onto the surface before assessing the strength of attachment puts some artificiality into the situation because this will inevitably increase the retention. However, as noted, the drying stage is essential, and the relative ease of removal can still be determined. In addition, in vivo, it is the most recalcitrant plaque and the most strongly retained cells that are of concern with regard to denture hygiene.

Some technical issues preclude direct comparison of the removal of the 2 microorganisms, although the different behaviors can be explained, as above. First, the methodologies used





8 Strength of attachment measurements. A, Carried out across. B, Along surface features by using *Streptococcus oralis* on surfaces of differing topographies by using increasing number of scans (S) with increasing applied force. By applying force across surface features, cells were more difficult to remove from LA and MA surfaces than HA surfaces. By applying force along surface features, cells could not be removed from LA surface. More cells remained on MA than HA surfaces. LA, Low abrasion; MA, medium abrasion; HA, high abrasion.

were not identical. Because of the rapid removal of the *C albicans* cells from the surface after wetting, the cells on the surface were dried between scans to encourage retention to obtain cell counts. However, *S oralis* was strongly attached and seemed to swell on wetting. It was not easy to obtain data using this microorganism. Comparing these results with those obtained by using other oral streptococci would be interesting and perhaps beneficial. An additional limitation is that the forces applied to the attached cells cannot be directly compared because the forces cannot be set so that all measurements start at the same point because they are affected by differences in the cantilever spring constant and environmental conditions, which vary from slide to slide. In addition, the scan sizes are different because the size of the microorganisms is so different (50 μm^2 *C albicans* and 10 μm^2 *S oralis*). Further, one of the problems of using the AFM in these types of studies is that, when adding a solution of cells onto the surface and determining the rate at which they bind is not possible because the cells interfere with the imaging. Thus, the cells were dried onto the surfaces before analysis. However, we found that the AFM is the best method to use to gain quantitative data of cell attachment to surfaces under static conditions. This work focused on cells attached onto a surface from a planktonic suspension; subsequent colonization and biofilm formation have not been addressed.

Finally, a number of technical difficulties need to be overcome when strength of attachment of microbial cells to polymer surfaces is measured with AFM. A highly charged surface repels the scanning cantilever tip, and, so, a high force has to be used initially to overcome the inertia of the electrostatic interactions of the surface. Although strength of attachment assays have previously been carried out on polymer surfaces.[24,25] this study is the first to use the AFM to measure the strength of attachment of oral species on denture acrylic surfaces. To the authors' knowledge, this is the first publication to compare the number and strength of attachment of organisms to surfaces. In terms of the removal of attached cells, it is the latter parameter that is of more concern. The AFM provides data on a scale that enables an assessment of the relationship between the dimension of the cell and the topographic features with which it is interacting.

CONCLUSIONS

This study reports the importance of surface feature size on cell attachment, retention, and putative subsequent hygiene issues. Linear features increased in

size from smooth to HA surfaces, and both types of cells were retained in greater numbers on the HA surfaces. After the force of attachment measurements, both types of cells were removed from the surfaces more easily when force was applied to the cells in a direction across the surface features instead of along the surface features. *S oralis* was harder to remove from the surfaces than was *C albicans*. Cell attachment was found to be related to cell size and, thus, the area of interaction between the cell and surface features. The findings reveal that even small abrasions may enhance cell attachment and retention on surfaces and reduce surface cleanability.

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